



Testing the prey naiveté hypothesis: Can native prey (*Astyanax ruberrimus*) recognize an introduced top predator, *Cichla monoculus*?

D. M. T. Sharpe · J. J. P. R. de Lira · G. E. Brown · M. E. Torchin · A. P. Hendry

Received: 19 December 2019 / Accepted: 19 September 2020 / Published online: 29 September 2020
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Abstract The prey naiveté hypothesis (PNH) posits that prey will often fail to recognize and respond to introduced predators with whom they do not share a co-evolutionary history. We tested this hypothesis by examining anti-predator behaviour in the native characid fish *Astyanax ruberrimus* in response to its main native (*Hoplias microlepis*) and introduced (*Cichla monoculus*) fish predators in Panama. We observed the behaviour of wild-caught *A. ruberrimus* from an invaded and uninvaded site following exposure to chemical stimuli from: (1) injured conspecifics, (2) the native predator, and (3) the introduced predator. We found, first, that *A. ruberrimus*

consistently responded to cues from injured conspecifics, suggesting that this species possesses an alarm signaling mechanism similar to that observed across Ostariophysan fishes. Second, *A. ruberrimus* responded to cues from their native predator, but only in one population, suggesting responses may be threat-sensitive. Third, *A. ruberrimus* lacking prior exposure to *C. monoculus* did not respond to cues from this predator, consistent with the PNH. In contrast, *A. ruberrimus* that have co-occurred with *C. monoculus* for several decades did respond to cues from this predator, suggesting that prior exposure to *C. monoculus* has led (either via local adaptation or learning) to acquired predator recognition. Overall, our findings are consistent with the PNH, although we cannot conclusively rule out alternate explanations for the observed differences between populations. Our work represents a first step towards understanding the role that behavioural naiveté may have played in the initial stages of this important tropical freshwater introduction.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10530-020-02369-4>) contains supplementary material, which is available to authorized users.

D. M. T. Sharpe (✉)
Worcester State University, Worcester,
MA, USA
e-mail: dsharpe@worcester.edu

D. M. T. Sharpe · J. J. P. R. de Lira · A. P. Hendry
Department of Biology, McGill University, Montréal,
QC, Canada

D. M. T. Sharpe · M. E. Torchin
Smithsonian Tropical Research Institute, Panama City,
Panama

G. E. Brown
Concordia University, Montréal, QC, Canada

Keywords Biological invasions · Prey naiveté · Chemical cues · Predator–prey interactions · Anti-predator behaviour · Peacock bass

Introduction

Predator introductions frequently lead to declines in the abundance and diversity of native prey, and these impacts tend to be particularly strong for terrestrial species on oceanic islands (Blackburn et al. 2004) and aquatic species in freshwater lakes (Hall and Mills 2000; Vitule et al. 2009). A leading hypothesis for this pattern is that because these isolated habitats tend to have few native predators, prey are evolutionarily naïve—and thus disproportionately vulnerable—to novel predators (Diamond and Case 1986; Cox and Lima 2006). In freshwater systems, geological barriers between watersheds limit the dispersal of both predators and prey, resulting in strong heterogeneity of predation regimes even at local scales. Thus, fishes are likely to be extremely vulnerable to novel predator archetypes with which they share no recent evolutionary history. For example, in East Africa, a diverse species flock of haplochromine cichlids radiated and flourished in Lake Victoria for tens of thousands of years completely isolated from large-bodied piscivores that were present elsewhere in the region (Lowe-McConnell 1993). When predatory Nile perch were introduced in the 1950s from nearby lakes Turkana and Albert, 40% of the endemic cichlid species disappeared in the span of only two decades (Ogutu-Ohwayo 1990; Witte et al. 1992; Kaufman and Ochumba 1993). This staggering loss in native diversity is frequently attributed to the assumed naiveté of the native cichlids *vis-a-vis* this novel predator. However, prey naiveté has been described as a “widely accepted but little studied ecological truism” (Cox and Lima 2006), and is only beginning to be tested empirically in the context of biological invasions (Shave et al. 1994; Kristensen and Closs 2004; Smith et al. 2008; Kovalenko et al. 2010; Dunlop-Hayden and Rehage 2011; Anton et al. 2016, 2020). Understanding the prevalence of prey naiveté, and the sensory and behavioural mechanisms that underlie it, is important for predicting the outcome of ongoing and future biological invasions. This is particularly true in tropical ecosystems, which hold the vast majority of the Earth’s freshwater biodiversity, yet remain greatly understudied (Lévêque et al. 2008).

Prey naiveté may manifest itself on multiple levels, from a failure to detect and respond to a novel predator, to mounting a behavioural defense that is inappropriate or ineffective (Banks and Dickman

2007; Carthey and Banks 2014). We focus here on the first level—recognition and response—as it is arguably the most critical in the sequence (Cox and Lima 2006). Prey are under strong selection to quickly and accurately detect predators (Lima and Dill 1990), and fishes have evolved sophisticated mechanisms to gauge predation risk based on a combination of visual, mechanosensory, and chemical cues (reviewed in Smith 1992; Chivers and Smith 1998; Kats and Dill 1998; Wisenden and Chivers 2006; Ferrari et al. 2010). These cues can range in specificity, which in turn can affect an animal’s ability to correctly recognize and respond to a novel threat. For example, many Ostariophysan fishes release alarm cues from their epidermal club cells when they are injured or killed during a predator attack, and these stimuli trigger a strong, innate fright response in nearby conspecifics (reviewed in Pfeiffer 1977; Chivers and Smith 1998). A general alarm signaling mechanism like this will increase the probability of detecting a broad range of potential threats, but will also increase the likelihood of repeatedly engaging in unnecessary and costly anti-predator behaviour. At the other extreme, some species are capable of learning to recognize more specific predator cues, such as the odour of a particular piscivore, or the odour of feces of a predator that has recently fed on conspecifics (reviewed in Brown 2003). A highly specific signaling mechanism like this will reduce the opportunity cost of unnecessary anti-predator responses, but will also increase the risk of failing to detect a potentially lethal novel threat.

Sih et al. (2010) provide a useful framework for predicting how interactions between native prey and novel predators may unfold, based on the specificity of cues that prey use to gauge risk, and the degree of similarity in cues between native and introduced predators. If prey rely primarily on predator-specific cues, but native and non-native predators produce different cues, then prey may fail to recognize and respond to a novel predator and may experience population declines. Conversely, if native and non-native predators happen to produce very similar cues; or if prey rely predominately on general cues, such as damage-released alarm cues, then native prey may successfully recognize and respond to an introduced predator and fare better.

We explored anti-predator behaviour in a native prey fish (*Astyanax ruberrimus*) in response to its main

native (*Hoplias microlepis*) and introduced (*Cichla monoculus*) fish predators in the Chagres watershed in Panama. *A. ruberrimus* are small-bodied, omnivorous, schooling characids that are common in streams, pools, rivers, lakes and reservoirs across Panama (Breder 1927; Zaret and Rand 1971; Angermeier and Karr 1983; Smith et al. 2004). They are native to Panama, and were historically one of the most abundant species in the Chagres watershed (Meek and Hildebrand 1916; Hildebrand 1938; Zaret and Paine 1973). Like other Ostariophysan fishes (Pfeiffer 1977), members of the genus *Astyanax* are thought to produce an alarm substance when attacked that triggers a fright response in conspecifics (Fricke 1987). The dominant native predator of *A. ruberrimus* in Panama is the dogfish, *Hoplias microlepis*—a nocturnal ambush predator that feeds primarily on characids (Breder 1927; Angermeier and Karr 1983; Kramer and Bryant 1995).

Peacock bass (*Cichla monoculus*) are large, highly-active, diurnal pursuit predators native to the Amazon Basin (Kullander and Ferreira 2006) that were introduced to the Chagres watershed in Panama for sport fishing in the 1960s (Zaret and Paine 1973). We propose that peacock bass represent a novel predator archetype (sensu Cox and Lima 2006) for native prey fishes in this ecosystem. They are phylogenetically distinct from native predators (Cichlidae vs. Erythrinidae), employ a different hunting mode (active pursuit vs. ambush), forage at a different time (diurnal vs. nocturnal), and are larger-bodied. Several lines of evidence suggest that they have strongly impacted native populations of *A. ruberrimus* in Panama. *A. ruberrimus* were locally extirpated following the establishment of peacock bass in Lake Gatun (Zaret and Paine 1973), and populations have not recovered over the past 45 years (Sharpe et al. 2017). Furthermore, *A. ruberrimus* are more than 20 times less abundant in areas where they co-occur with peacock bass relative to areas that have not been invaded (Sharpe et al. 2017).

The overarching goal of our study was to explore whether behavioural naiveté may help explain the apparent vulnerability of *A. ruberrimus* to the introduced peacock bass. To address this goal, we conducted a series of laboratory experiments to test the following questions: (1) Do *A. ruberrimus* respond to conspecific alarm cues, and if so, what is the qualitative nature of this anti-predator response? (2)

Do *A. ruberrimus* respond to cues from their native (*H. microlepis*) predator? (3) Do *A. ruberrimus* respond to cues from their introduced (*C. monoculus*) predator, and if so, do these responses differ between invaded versus uninvaded populations? The first question was meant both to ascertain whether *A. ruberrimus* is capable of recognizing and responding to chemical alarm cues in general, and to develop a baseline description of their anti-predator behaviour. This was an important preliminary step because there has been no published work either on the sensory ecology of this species, or on their behaviour (other than some brief observations of their feeding behaviour in the wild almost a century ago, Breder 1927). Furthermore, understanding how native species respond to conspecific alarm cues is relevant to predicting how they might fare in the face of a novel predator, because these general responses can complement species-specific responses (Sih et al. 2010). The next two questions were meant to specifically test predictions of the prey naiveté hypothesis, namely that *A. ruberrimus* from both populations would readily respond to cues from the native *H. microlepis* (question 2) but that individuals from the uninvaded population would fail to respond to the introduced *C. monoculus* (question 3).

Methods

Study sites and fish collection

Astyanax ruberrimus were collected with beach seines and cast-nets from two sites in the Chagres watershed, in Central Panama: one in which the invasive predator is absent (Quebrada Juan Grande; uninvaded locality) and one in which the invasive predator is present (Rio Chagres, invaded locality). Quebrada Juan Grande is a small stream draining lowland tropical rainforest. No introduced species have been recorded at this site, and the fish community is dominated by native characins (Angermeier and Karr 1983, Sharpe, unpubl. data). The only piscivore at this site is the native dogfish, *Hoplias microlepis* (Angermeier and Karr 1983). Rio Chagres is a large river that drains into Lake Gatun and the Panama Canal. Peacock bass invaded this site in 1967 after escaping from a nearby fishing pond (Zaret and Paine 1973). The native *H. microlepis* is also present at this site (Valverde, M., unpubl. data). *Cichla monoculus*

and *H. microlepis* were collected by hook and line from Lake Gatun, Panama. All fish were collected between March and May of 2016, with permission from Panama's Ministry of the Environment, MiAmbiente (Permit # SE/AP-40-15).

Fish care

After capture, we transported all fish in large coolers filled with aerated lake water to our laboratory at the Smithsonian Tropical Research Institute. Prior to the experiments, fish were maintained in large (265L) outdoor stock tanks in continuously filtered, dechlorinated tap water at 24 °C on a 12:12 light:dark cycle. *A. ruberrimus* were acclimated to these laboratory conditions for at least five days prior to testing. *A. ruberrimus* were fed commercial flake food (Tetraamin®) twice daily ad libitum. Predators (*C. monoculus* and *H. microlepis*) were fed live fish (*A. ruberrimus*), except when being held for odour extraction (see below). All protocols for the handling of live organisms were approved by STRI's Institutional Animal Care Committee (Protocol # 2016-0224-2019).

Preparation of conspecific and predator cues

To prepare the conspecific (*Astyanax*) alarm cue, we used skin extracts from euthanized *A. ruberrimus*. Skin tissue was used because conspecific alarm cues are believed to originate from special club cells in the epidermis of Ostariophysi (Pfeiffer 1977). We first removed a total of 171.3 cm² of skin fillets from 17 euthanized individuals of both sexes, eight from the Juan Grande river (SL: 47.62 ± 13.36 mm) and nine from the Chagres river (SL: 51.28 ± 8.83 mm). Dissected skin fillets were transferred to a glass beaker containing 100 mL of chilled distilled water, and blended to homogenize the mixture. We then removed 30 mL aliquots with a pipette and stored them in Whirl-Pak® bags at -20 °C until needed.

To prepare the two predator cues, we used water from tanks containing an individual predator. Each predator (three *H. microlepis*, SL: 21–24 cm and three *C. monoculus*; SL: 20–25 cm) was transferred to a 30 L tank immediately upon arrival to the laboratory. Individuals were kept in this holding tank for 48 h under food deprivation, after which time we obtained a 10L water sample. We then mixed the three samples

from each predator species, and stored the mixtures in 30 mL aliquots in Whirl-Pak® bags at -20 °C until needed. By mixing the samples from multiple individuals (the same procedure used for the alarm cue preparation), we sought to ensure that there would not be an individual donor bias in our experiments (Brown et al. 2013).

Experimental protocol

We implemented a 2 × 4 experimental design in which *A. ruberrimus* from the uninvaded (Juan Grande) and invaded locality (Chagres) were exposed to four different stimuli (treatments): distilled water (Control), a conspecific alarm cue (*Astyanax*), a native predator cue (*H. microlepis*), and an introduced predator cue (*C. monoculus*). Although the order of trials was randomized, we were occasionally limited by the availability of fish from each source population. Therefore, we adjusted the number of trials that were conducted per day so that fish from both populations were tested at the same time of day in the majority of cases. We aimed for 10 replicate trials per treatment × population combination, but some trials had to be discarded, usually because aggressive interactions among the fish resulted in one individual being injured or killed during the acclimation period or trial. Even after excluding these trials, we cannot rule out the possibility that stress from aggressive intraspecific interactions may have influenced behavioural responses to experimental cues in some cases. In total, we analyzed data from 70 trials, with the number of independent replicates of each population × treatment ranging from six to eleven (Table S1).

Each trial consisted of the observation of three roughly size-matched individuals over a 10 min period that included the injection of one of the four experimental stimuli at the 5 minute mark. In general, individual fish were only tested once; however, on five occasions we did re-use fish that had been previously used in a control trial. We analyzed our data both including and excluding these five trials with re-used fish, and results were almost identical either way (not shown), so we present results from the full data-set. Prior to each trial, we transported *A. ruberrimus* from the outdoor holding tanks to an indoor laboratory where the experimental tanks were kept. The room temperature was 22 °C and illumination was provided by fluorescent light bulbs and natural diffuse light.

Fish were transported in 7 L tanks, were acclimated for 30 min, and then were transferred to the experimental tanks, where they were allowed to acclimate for at least 20 h prior to testing.

Experimental trials were conducted in 21 L ($32 \times 31 \times 21$ cm) glass aquaria filled with chilled, dechlorinated tap water and 2 cm of sand as a substrate. Each tank contained a small electric filter and a single airstone, to ensure that water was continuously aerated. Each tank also had a white PVC drain tube in one corner, which (although this was not our original intent) the fish often chose to hide behind. For this reason, we chose to examine shelter use as one of the variables in our study (see below). All tanks were covered on three sides with black plastic to prevent visual disturbance and communication between fish in different tanks, and with a removable, black barrier on the remaining side. The top of each tank was covered with mesh to prevent fish from escaping. We also drew horizontal lines on the front of the tank to facilitate estimation of vertical area use.

Test fish were fed flake food 15 min prior to testing. By doing so, we not only provided foraging opportunities to the fish during the video recordings, but also guaranteed that our observations were not confounded by food deprivation (Brown and Smith 1996). To begin a trial, we removed the barrier and started the video recording. After 5 min of baseline observation, we injected the stimulus (either distilled water, *Astyanax* cue, *H. microlepis* cue or *C. monocus* cue) into the tank. To inject the stimulus, we used a syringe connected to a length of transparent tubing that was placed in the back of the tank. We slowly injected 10 mL of stimulus through the tubing, and then flushed it into the tank with tank water that had been set aside earlier. It usually took between 30 s and 1 min for the stimulus to be fully injected and flushed, after which time, we observed and recorded the fish for another 5 min. All trials were recorded in the morning (between 8:00 and 12:00 am) with a Nikon J1 video camera. Tanks were completely drained of water and rinsed in between trials to prevent any residual cues from influencing subsequent trials.

Behavioural observations

From the video recordings, we quantified the following six behavioural metrics: (1) vertical area use, (2) shoaling, (3) shelter use, (4) time spent motionless, (5)

dashing, and (6) freezing—all of which have been previously described as part of the fright response of other small fishes (Chivers and Smith 1998). Preliminary inspection of the videos suggested that *Astyanax* tended to exhibit extremely brief/transient responses to stimulus injection, so we chose to restrict our analysis of these variables to the following three time periods: 1 min prior to stimulus injection, 1 min during stimulus injection, and 1 min post-stimulus.

Vertical area use, shoaling and shelter use were scored every 15 s. Vertical area use was the sum of the vertical position of each of the three fish (1 if a fish was in the bottom half of the tank, and 2 if it was in the top half of the tank), and overall scores ranged from 3 (all fish close to the substrate) to 6 (all fish close at the surface). Shoaling was quantified using a shoaling index (Marcus and Brown 2003), and ranged from 1 (no fish within one body length of each other) to 3 (all fish within one body length of each other). Shelter use was the number of fish that were motionless (for at least 5 s) near the substrate either in a corner of the tank, or within one body length of the PVC drain pipe. Shelter scores ranged from 0 (no fish sheltering) to 3 (all fish sheltering either in a corner or behind the pipe). For each minute (pre and post-stimulus injection), we recorded the total time that each individual fish spent motionless, and then summed these values for the shoal. Dashing and freezing were assessed as categorical (Y/N) variables for each time period. Dashing was defined as at least one individual exhibiting several seconds of “very rapid, apparently disoriented swimming” (Lawrence and Smith 1989). Freezing was defined as at least one individual “ceasing movement and remaining motionless on the substrate for at least 30 s uninterrupted” (Brown et al. 1997).

Statistical analysis

First, we sought to assess variation in baseline (pre-stimulus) behaviour. To do so, for the first four variables (vertical area use, shoaling, shelter use, and time spent swimming), we tested for variation in pre-stimulus behavioural scores across populations and treatments using separate non-parametric Kruskal–Wallis tests. Overall, as expected, pre-stimulus behaviour did not differ across populations or treatments (results not shown); except in one case: pre-stimulus vertical area use differed across treatments in

the JG population ($\chi^2 = 10.762$, $p = 0.013$). Given this result, we proceeded to analyze behavioural responses to experimental stimuli using proportional rather than raw difference scores to standardize for this variation in baseline conditions.

Proportional difference scores were calculated for each tank as follows: Relative change in behaviour = (mean score *post-stimulus* – mean score *pre-stimulus*)/mean score *pre-stimulus*.

These difference scores then served as the dependent variables in subsequent analyses. We examined the distribution of difference scores within each population and treatment, and tested for normality for each distribution using the Shapiro–Wilk test. With one exception, distributions were *not* normal (results not shown). Therefore, we proceeded to test for variation in difference scores across treatments using separate non-parametric Kruskal–Wallis tests for each behavioural metric in each population. We did not correct for multiple comparisons across these separate Kruskal–Wallis tests ($n = 16$) because we judged the cost of a false positive to be lower than the cost of a false negative. In cases where we did find a significant overall treatment effect, we used Dunn’s post hoc multiple comparison test to evaluate differences between each treatment and the control. We used an alpha of 0.05 to determine statistical significance.

For categorical variables (presence or absence of dashing and freezing behaviour), we tallied up the frequency of the behaviour in each of the three time periods (pre, during and post-stimulus), and calculated the number of trials in which the behaviour decreased, did not change, or increased relative to pre-stimulus. We then used Chi Square tests to determine whether the frequencies of these three outcomes differed in each treatment relative to the control. All analyses were conducted in R (R Core Team 2019).

Results

Astyanax ruberrimus from the uninvaded population (Juan Grande) did not alter their vertical area use, shoaling, shelter use, time spent motionless, dashing or freezing behaviour either during or after the injection of either the native (*H. microlepis*) or introduced (*C. monocolus*) predator cue (Fig. 1, Tables 1, S2, S3). In contrast, in response to the conspecific *Astyanax* alarm cue, we observed an

increase in dashing during the minute that the cue was being injected ($\chi^2 = 4.904$, $p = 0.027$, Fig. 2A, Table S2). There was no difference; however, in any of the other measured behavioural metrics either during or after injection of the conspecific alarm cue (Table 1, S2, S3).

In the invaded population (Chagres), *A. ruberrimus* showed a response to all three experimental cues (Tables 1, 2). In response to the conspecific *Astyanax* alarm cue, we observed an increase in dashing *during* the injection of the stimulus ($\chi^2 = 7.378$, $p = 0.025$, Fig. 2b), as well as a downwards shift in the water column ($Z = -2.61$, $p = 0.005$, Fig. 1b) and an increase in shoaling ($Z = 2.89$, $p = 0.002$, Fig. 1d) *post-stimulus*. There was no change in shelter use, time spent motionless or freezing either during or post-stimulus for the conspecific cue. In response to the native predator cue (*H. microlepis*), *A. ruberrimus* showed a decrease in shelter use *during* the injection of the stimulus ($\chi^2 = 2.575$, $p = 0.005$, Fig. 1f). We also observed a trend towards an increase in shoaling *post-stimulus*, but this was not statistically significant ($Z = -1.36$, $p = 0.086$, Fig. 1d). There was no change in vertical area use, time spent motionless, dashing or freezing either during or post-stimulus for the native predator cue. In response to the introduced predator cue (*C. monocolus*), we observed a decrease in shelter use *during* the injection of the stimulus ($\chi^2 = 2.390$, $p = 0.008$, Fig. 1f), and an increase in shoaling *post-stimulus* ($Z = 1.816$, $p = 0.035$, Fig. 1d). There was no change in vertical area use, time spent motionless, dashing or freezing either during or post-stimulus for the introduced predator cue.

Discussion

The introduction of the peacock bass in Lake Gatun is a classic example of a freshwater introduction that has had substantial impacts on the native fish community (Zaret and Paine 1973; Sharpe et al. 2017). Here, we sought to explore whether behavioural naiveté could explain the vulnerability of *A. ruberrimus*—a previously dominant member of the native littoral fish community that experienced between 80 and 100% declines in abundance post-introduction (Sharpe et al. 2017). Specifically, we asked whether *A. ruberrimus* respond to different types of chemical cues (conspecific alarm cues, cues from the native the introduced

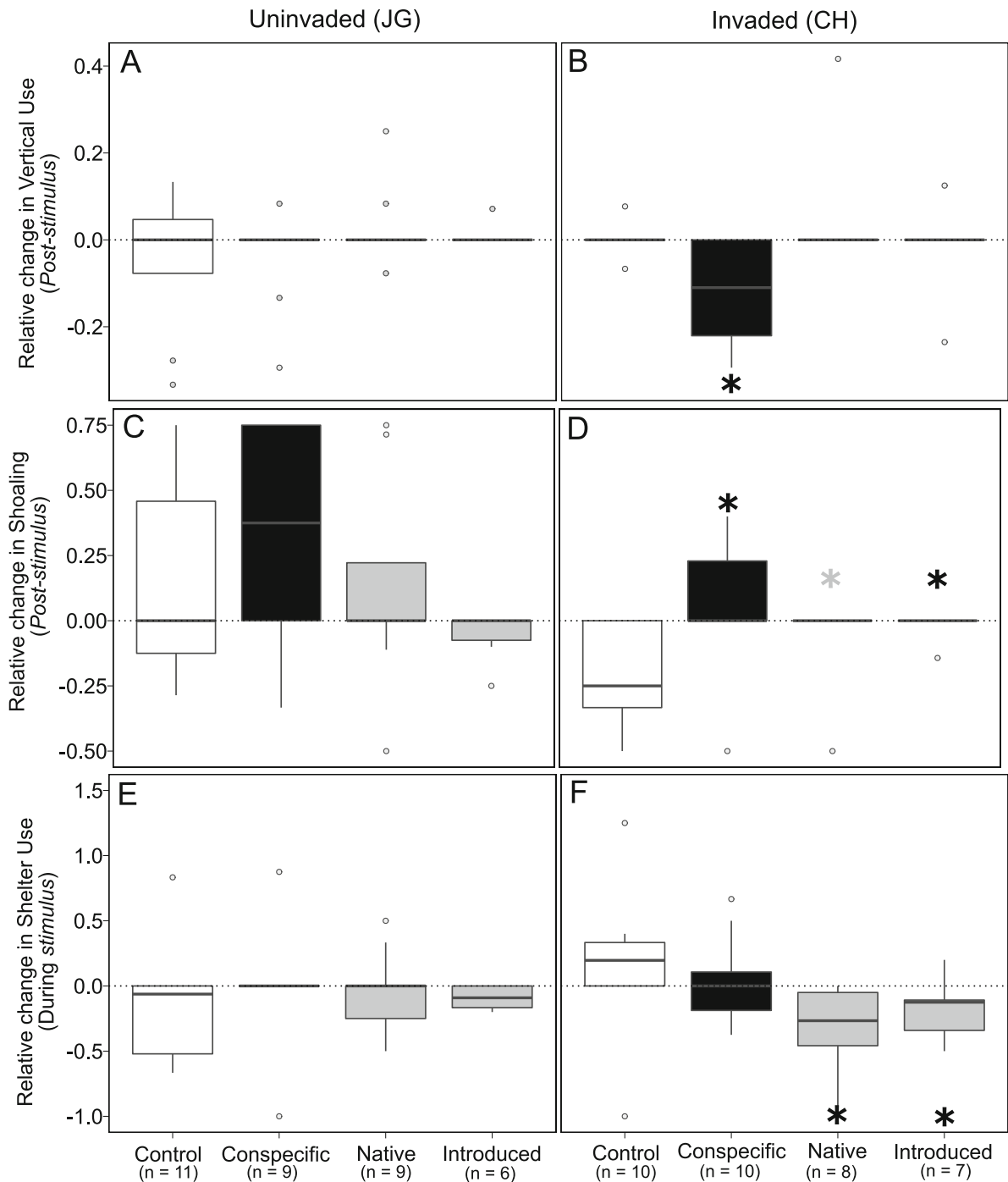


Fig. 1 Relative change in vertical area use (Panels **a**, **b**), shoaling (Panels **c**, **d**), and shelter use (Panels **e**, **f**) either *during* or *post* stimulus, in the uninvaded (Juan Grande, left) and invaded (Chagres, right) population. Data shown are proportional difference scores, which compare the mean behavioural score for the entire shoal either during or post stimulus relative to pre-stimulus (see Methods for details). Boxplots show the

median (heavy line), the interquartile range (box), the range excluding outliers (whiskers), and outliers (points whose value is greater than 1.5 times the interquartile range, light grey points). Symbols indicate treatments where the change in the behaviour was significant (black asterisk, $p < 0.05$) or marginally significant (grey asterisk, $p < 0.10$) relative to the control; see Table 2 for details

Table 1 Response variables were proportional difference scores, which compare the mean behavioural score for the entire shoal either *during* or *post* stimulus relative to *pre*-stimulus (see Methods for details)

Pop	Response	Period	Overall effect		Direction relative to control		
			χ^2	p	Con-specific Cue	Native Predator Cue	Introduced Predator Cue
JG	Vertical use	During	1.44	0.697			
		Post	1.33	0.723			
	Shoaling	During	5.22	0.156			
		Post	3.51	0.319			
	Shelter use	During	1.38	0.709			
		Post	2.58	0.461			
	Time still	During	4.33	0.228			
		Post	4.53	0.209			
	Freezing	During					
		Post					
Dashing	During			+			
	Post						
CH	Vertical use	During	5.28	0.153			
		Post	10.95	0.012	-		
	Shoaling	During	2.95	0.399			
		Post	8.63	0.035	+	(+)	+
	Shelter use	During	9.28	0.026		-	-
		Post	2.90	0.407			
	Time still	During	5.43	0.143			
		Post	1.43	0.699			
	Freezing	During					
		Post					
Dashing	During			+			
	Post						

For vertical use, shoaling, shelter use, and time still, overall treatment effects were first evaluated using non-parametric Kruskal–Wallis tests, followed by Dunn’s post hoc multiple comparison tests (see Table 2 for details). Significant ($p < 0.05$) overall treatment effects are shown in bold. For freezing and dashing, see Tables S2 and S3 for detailed statistical results. The final three columns show the direction of any significant effects for each treatment relative to the control: empty grey cells represent cases where the behaviour did not differ between the treatment and the control, green cells with a “+” symbol indicate cases where the behaviour *increased* significantly over time in the treatment relative to the control, and red cells with a “-” symbol indicate cases where the behaviour *decreased* significantly over time in the treatment relative to the control. Note that a single marginally significant result is indicated by a plus symbol in parentheses

predator); and whether responses to these cues differ between uninvaded and invaded localities. We found that *A. ruberrimus* from both the uninvaded (Juan Grande) and invaded (Chagres) population responded to the conspecific alarm cue. With respect to predator cues, *Astyanax ruberrimus* from Juan Grande showed no response to cues from either the native (*H. microlepis*) or introduced (*C. monoculus*) predator; whereas their counterparts from the Chagres responded to both. Below we discuss these findings

in relation to the three questions we posed at the outset, and then consider the extent to which prey naiveté may have played a role in this particular invasion, and more broadly.

Do *A. ruberrimus* respond to conspecific alarm cues?

We found that *A. ruberrimus* exhibited a clear behavioural response to conspecific skin extract,

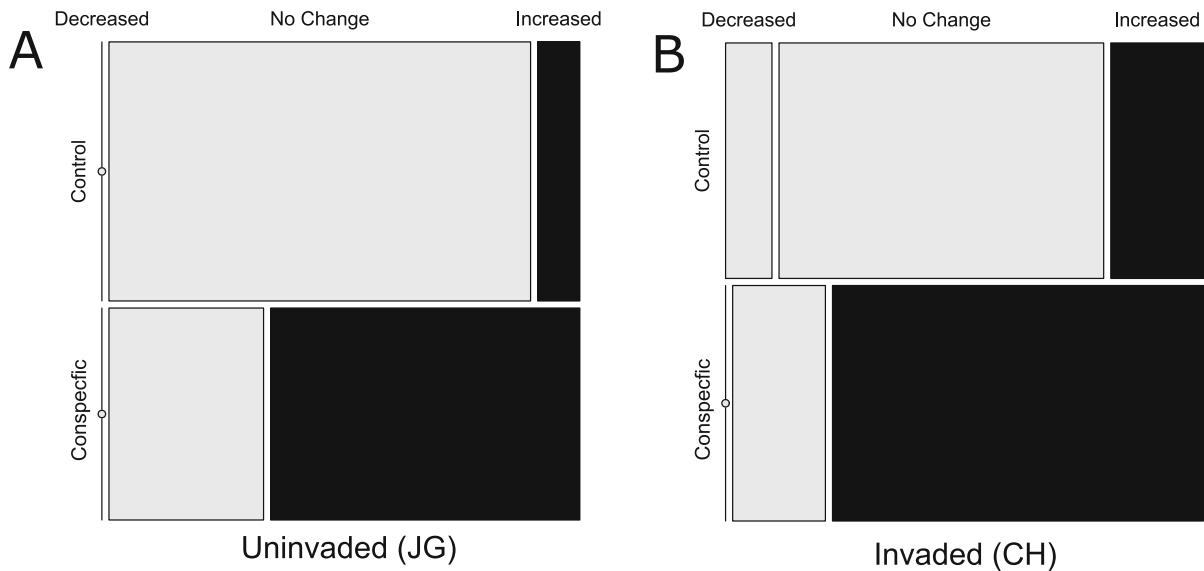


Fig. 2 Mosaic plots showing the proportion of trials in which dashing either decreased, did not change, or increased during the injection of the stimulus, relative to pre-stimulus. The relative frequencies of these three outcomes differed significantly

between the control and the conspecific cue for both the uninvaded (JG, Panel a, $\chi^2 = 4.904, p = 0.027$) and invaded (CH, Panel b, $\chi^2 = 7.378, p = 0.025$) populations

Table 2 Results of post hoc non-parametric Dunn tests examining differences between each treatment versus the control

Pop	Response	Period	Conspicific versus control		Native versus control		Introduced versus control	
			Z	p	Z	p	Z	p
CH	Vertical Use	Post	- 2.611	0.005	- 0.453	0.325	- 0.120	0.452
CH	Shoaling	Post	2.886	0.002	- 1.363	0.086	1.816	0.035
CH	Shelter Use	During	1.040	0.149	2.575	0.005	2.390	0.008

Post-hoc tests were only conducted in cases where Kruskal–Wallis tests revealed a significant overall treatment effect (see Table 1). Significant (< 0.05) differences are shown in bold; marginally significant (< 0.10) differences are shown in italics

consistent with reports from the congeneric *Astyanax bimaculatus* and *A. fasciatus* (Pfeiffer 1977; Fricke 1987). Conspecific alarm cues elicited a response in both populations, despite variation in past exposure to predators, consistent with the view that this mechanism is innate and highly-conserved within Ostario-physan fishes (Chivers and Smith 1998).

The behavioural response to conspecific alarm cues included dashing during the injection of the stimulus (observed in both populations), and increased shoal cohesion and a shift toward the substrate post-stimulus (observed in the Chagres population only). Dashing (also referred to as “zigzagging”) has been observed in other fishes, and has been variously interpreted as

vigilant/exploratory behaviour (Kovalenko et al. 2010), or as a means to visually alert conspecifics to danger (Fricke 1987). The fact that dashing was observed during but not after the injection of the stimulus is consistent with the idea that it may represent some sort of exploratory/inspection behaviour in this species.

Shoaling is a common anti-predator behaviour in small prey fishes, and has been shown to be both adaptive and heritable (Magurran 1990a, b). Shifts in vertical distribution are a common avoidance behaviour, and are thought to reflect the habitat use of predators, with prey shifting upwards in the water column in response to benthic predators (e.g., Dunlop-

Hayden and Rehage 2011), and downwards in response to pelagic predators (e.g., Lozada et al. 2000). The downwards shift in vertical distribution in the Chagres population is more consistent with the expected escape response to *C. monoculus* (a pelagic predator) than to *H. microlepis* (a benthic predator), and may reflect the stronger predation pressure imposed by the former in this invaded habitat. Ultimately however, staged encounters between live prey and their native and introduced predators would be the only way to demonstrate whether assumed “anti-predator” behaviours actually increase survival.

Do *A. ruberrimus* respond to cues from their native (*H. microlepis*) predator?

We expected that *A. ruberrimus* from both populations would respond strongly to chemical cues from their native predator, *H. microlepis*, with whom they share a long co-evolutionary history. Contrary to this expectation, *A. ruberrimus* from Juan Grande did not alter their behaviour in any way following exposure to chemical cues from *H. microlepis*. In contrast, *A. ruberrimus* from the Chagres responded to the *H. microlepis* cue by significantly decreasing shelter use during stimulus injection.

There are two possible explanations for the lack of a response in the Juan Grande population. First, it is possible that the cues were simply not detected. Many fishes are sensitive to the concentration of chemical stimuli, and will not respond below a certain threshold (Lawrence and Smith 1989; Lozada et al. 2000; Marcus and Brown 2003). However, this seems unlikely, as we followed well-established protocols for the preparation of our predator stimuli (Brown et al. 1997), and we used the same batch for both populations. If the Chagres population was able to detect the cues, it seems most reasonable to assume that the Juan Grande population could as well.

A second, and more likely, possibility is that the native predator cue was detected in both populations, but only elicited a behavioural response in the Chagres population. Predator avoidance behaviours have obvious fitness benefits, but also carry significant costs in terms of energy expenditure and lost time for foraging, courtship and parental care. Thus, animals should be under strong selection to accurately assess risk, and make threat-sensitive behavioural decisions that balance the risk of mortality against these opportunity

costs (Helfman 1989; Lima and Dill 1990). There is a wealth of evidence that prey are capable of integrating information from multiple cues in a very sophisticated manner, and will adjust the intensity of their response to the perceived threat level (reviewed in Lima and Dill 1990). For example, western mosquitofish *Gambusia affinis* modulate avoidance and inspection behaviours based on predator size, diet, and hunger level, responding more strongly to large, hungry sunfish *Lepomis cyanellus* that have recently fed on conspecifics (Smith and Belk 2001). Similarly, fathead minnows *Pimephales promelas* respond to pike *Esox lucius* that have been fed conspecifics, but not heterospecifics (Mathis and Smith 1993). Fish also often respond more strongly when chemical and visual cues are combined (e.g., Kovalenko et al. 2010), and—unsurprisingly—when exposed to a live predator versus artificially injected chemical cues (e.g., Dunlop-Hayden and Rehage 2011).

In our study, *A. ruberrimus* from the uninvaded population, that live under a lower threat of predation in the wild, after sensing *H. microlepis* cues alone may simply have judged predation risk to be too low to warrant a costly response. In contrast, *A. ruberrimus* from the invaded population, who live constantly under a higher risk of predation in the wild, may have a lower threshold for response. We believe that the significant decrease in shelter use during the stimulus injection that we observed in this population may represent an exploratory behaviour. As soon as individuals detected the cue, they left their previous resting positions in the corners of the tank and swam around in the open water, perhaps trying to inspect the potential threat. The fact that there was no clear, sustained anti-predator response after this brief inspection period suggests that they may have judged the threat to be minimal.

Future research should explore anti-predator behaviour in *A. ruberrimus* under a broader range of experimental conditions, which would include manipulating the strength of cues (e.g., different concentrations of chemical stimuli), type of cues (visual, chemical vs. live predator), and predator attributes (size, diet). Alternative experimental set-ups (e.g., with a live predator in a mesh enclosure, or with continuous flow-through from an adjacent tank containing a predator) would be helpful to understand how *A. ruberrimus* from both populations respond to native predator stimuli that are more realistic, more

concentrated and/or delivered over longer time periods. Overall, we interpret the lack of response to the native predator cue in the Juan Grande population with caution, as it is difficult to determine whether this reflected a true lack of recognition, or was the product of threat-sensitive behaviour (Chivers and Smith 1998).

Do *A. ruberrimus* respond to cues from their introduced (*C. monoculus*) predator, and if so, do responses differ between invaded *versus* uninvaded populations?

The prey naiveté hypothesis predicts that prey that lack a recent co-evolutionary history with a novel predator should fail to recognize and respond to that predator. Thus, we expected that *A. ruberrimus* from the uninvaded site (Juan Grande) would fail to respond to chemical cues from the introduced *C. monoculus*. This was indeed what we observed: *A. ruberrimus* from Juan Grande showed no behavioural response whatsoever to cues from *C. monoculus*. This is consistent with the weak or absent anti-predator behaviour observed in predator-naïve populations, for example fishes reared in the lab (e.g., European minnows *Phoxinus phoxinus* (Magurran 1989)), or collected from wild populations lacking piscivores (e.g., fathead minnows (Mathis et al. 1993)).

In contrast, *A. ruberrimus* from the invaded population (Chagres) did respond to the *C. monoculus* cue, displaying a significant decrease in shelter use during the stimulus injection, and an increase in shoal cohesion *post*-stimulus. As explained above, we believe this decrease in shelter use during the stimulus injection may represent an initial exploratory response to the cue, where individuals leave their previous positions in the corners to move around the tank and inspect the stimulus. After this inspection period, they increased shoal cohesion, potentially indicating that they judged the stimulus to be a threat. We note that the Chagres population appeared to respond more strongly to the introduced predator cue (inspection followed by shoaling) than to the native predator cue (inspection only). This may reflect the fact that *C. monoculus* may pose a greater predation threat, given that in Lake Gatun they are both larger (mean \pm SD (cm): 27.58 \pm 5.32 vs. 20.21 cm \pm 1.25 for mature *C. monoculus* (n = 207) and *H. microlepis* (n = 15),

respectively; Sharpe, *unpl. data*) and more abundant (Sharpe et al. 2017).

The behavioural response that we observed in the Chagres population may reflect either learning or local adaptation, or a combination of the two. Naïve populations appear to be capable of learning to recognize a previously-unfamiliar predator very quickly (reviewed in Brown 2003). For example, in laboratory experiments, European minnows (Magurran 1989), fathead minnows (Chivers and Smith 1994) and brook trout *Salvelinus fontinalis* (Mirza and Chivers 2000) learned to recognize novel predators after a single exposure in which the predator cue was paired with an alarm cue. In the wild, previously-naïve fathead minnows learned to recognize chemical and visual cues only a few days after pike were experimentally introduced to their pond (Brown et al. 1997). In other cases, there is good evidence that inter-population variation in behaviour is heritable, and reflects local adaptation to divergent predator regimes. For example, in Trinidadian guppies, *Poecilia reticulata*, populations that have evolved under high versus low predation show heritable differences in schooling behaviour (Seghers 1974; Magurran et al. 1992; Huizinga et al. 2009). Overall, we suspect the heightened anti-predator behaviour observed in the Chagres reflects an adaptive response (either genetic or plastic) not only to the presence of the introduced *C. monoculus*, but also to generally higher predation levels. For example, the native *H. microlepis* grows to much larger body sizes here, and appears to be more abundant, than in small streams like Juan Grande (D. Sharpe, *pers. obs.*). Further work is needed to explore the extent to which intraspecific variation in behaviour in *A. ruberrimus* may reflect adaptation to local predation regimes over generational timescales versus learning that occurs during ontogeny.

To what extent might naiveté in *A. ruberrimus* have contributed to the declines observed following the introduction of *C. monoculus*?

Our experiments showed that *A. ruberrimus* with no prior experience of *C. monoculus* (Juan Grande population) failed to respond to cues from this novel predator. These results are in agreement with the predictions of the prey naiveté hypothesis, and suggest that naiveté may have contributed to the dramatic (> 90%) declines that were observed in *A. ruberrimus*

in Lake Gatun in the first year following the introduction (Zaret and Paine 1973). We should emphasize; however, that expectations of the PNH were not unambiguously supported here, because *A. ruberrimus* from Juan Grande failed to respond to cues from both the introduced and native predator. Therefore, we cannot be sure whether individuals from this population failed to recognize *C. monoculus* as a threat (as predicted by the PNH), or simply exhibited muted anti-predator behaviour overall.

In contrast, we observed that in the Chagres population, *A. ruberrimus* that have now co-occurred with *C. monoculus* for 49 years (~ 25 generations) responded to *C. monoculus* cues with typical anti-predator behaviour (inspection followed by increased shoaling). A recent meta-analysis by Anton and colleagues (2020) found that native prey typically require much more time—at least 215 generations on average—to recognize introduced predators. The comparably rapid acquisition of predation recognition in *A. ruberrimus* that we observed here may reflect either rapid evolution (perhaps due to particularly strong selection in this case) and/or learning. Our findings are consistent with those of other studies that have found that even when native prey are initially naïve, they often learn to recognize introduced predators relatively quickly after contact (Kiesecker and Blaustein 1997; Kristensen and Closs 2004).

Why then, do *A. ruberrimus* remain at such low abundance wherever they co-occur with *C. monoculus* today, almost half a century post-invasion (Sharpe et al. 2017)? Predator recognition is only the first step in an anti-predator response, and it is quite possible that even if *A. ruberrimus* have evolved/learned to recognize and respond to *C. monoculus* over time, their existing behavioural repertoire could be inappropriate for surviving an encounter with this novel predator. Or, their responses may be appropriate, but ultimately inadequate, i.e. *A. ruberrimus* remains “outgunned” by the superior speed, agility or foraging prowess of this novel predator (Banks and Dickman 2007; Carthey and Banks 2014).

Another relevant finding was that *A. ruberrimus* from both populations consistently responded to conspecific alarm cues. This arguably might predispose them to respond to any actively foraging predator, and thus be more likely to survive a first encounter with a novel piscivore (Sih et al. 2010). However, we suspect that damage-released alarm cues

might not be equally effective for alerting *A. ruberrimus* to the presence of actively foraging *H. microlepis* versus *C. monoculus* in a natural context, given their very different morphologies and hunting strategies. *H. microlepis* have large, sharp teeth (Meek and Hildebrand 1916), and Breder (1927) describes the foraging behaviour as follows: “Several times these voracious fish were actually seen to strike into schools of *Astyanax* and catch one, always holding it crosswise in the mouth before turning its head first for swallowing.” Manipulating prey in this way would presumably lead to more mechanical damage to the skin, and likely release more alarm pheromones. In contrast, *C. monoculus* have very small teeth (Kullander and Ferreira 2006), use ram/suction feeding (Norton and Brainerd 1993), and swallow their prey whole in a single gulp (D. Sharpe, *pers. obs.*), which might release less alarm pheromone.

Naiveté and the ecology of the freshwater invasions

The prey naiveté hypothesis is a logical explanation for why isolated faunas appear to be more vulnerable to introduced predators, particularly those which represent novel archetypes (Ricciardi and Atkinson 2004; Cox and Lima 2006; Anton et al. 2020). Our work represents a first step towards understanding the role that behavioural naiveté may have played in one important tropical freshwater introduction—that of the peacock bass in Panama. Our findings corroborate those of several other recent studies of invaded systems that suggest that naiveté may play an important role in the initial stages of an invasion, but also that recognition can be acquired over time (Kiesecker and Blaustein 1997; Kristensen and Closs 2004).

This suggests that a simple lack of response (Level 1 naiveté, *sensu* Banks and Dickman 2007) may not be the only factor driving sustained declines of native prey. Other aspects of naiveté, such as the nature of the anti-predator response itself, may be more important in the long-term. For example, tactics or defenses that function well with a co-evolved predator may be completely inappropriate (Level 2 naiveté) or ineffective (Level 3 naiveté) in the face of a novel predator (Banks and Dickman 2007; Carthey and Banks 2014). Interactions with other stressors may also be important. For instance, freshwaters have historically been

focal points of human settlement and impacts (Dudgeon et al. 2006). Thus, the vulnerability of lacustrine faunas to invasion may reflect not just evolutionary naïveté, but also interactions with other stressors. For example, in East Africa's Lake Victoria, eutrophication, hypoxia and over-fishing undoubtedly contributed to the declines of the haplochromine cichlids following the Nile perch introduction (Chapman et al. 2008). In the Neotropics, most of the strong impacts of peacock bass have been observed in reservoirs (Zaret and Paine 1973; Latini and Petrere 2004; Pinto-Coelho et al. 2008; Pelicice and Agostinho 2009; Menezes et al. 2012; Sharpe et al. 2017)—artificial habitats that lack many of the natural spatial and temporal refugia that native prey rely on (Kovalenko et al. 2010). Greater integration of laboratory-based behavioural experiments with field studies conducted at broad spatial scales is needed to understand these interactions, especially in understudied tropical lakes.

Acknowledgements We gratefully acknowledge M. Valverde, V. Bravo, C. Bonilla, J. Prevost, C. Schloeder, L.F. De León, and the staff of STRI's Naos Laboratories for valuable field and laboratory assistance and logistical support. We thank Associate Editor Angela Chuang and the three anonymous reviewers whose constructive comments greatly improved an earlier version of this manuscript. We thank Panama's Autoridad del Canal (ACP) and MiAmbiente for permission to collect fish as part of this study (Permit # SE/AP-40-15). Funding was provided by NSERC (NSERC CREATE in Biodiversity Ecosystem Services and Sustainability, BESS), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brazil (Science Without Borders Scholarship to JJPRL, Grant # 13317/13-0), the Smithsonian Tropical Research Institute, and the Sistema Nacional de Investigadores de Panama (to DMTS).

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